

An integrative description of *Mesobiotus ethiopicus* sp. nov. (Tardigrada: Eutardigrada: Parachela: Macrobiotidae: *harmsworthi* group) from the northern Afrotropic region

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Abstract: A new species of the *Mesobiotus harmsworthi* group is described from Ethiopia. An integrative taxonomy approach was applied by combining morphological and morphometric analyses—imaging under phase contrast and scanning electron microscopy with molecular analysis (18S rRNA, 28S rRNA and COI markers)—to cross-verify the status of the population as a new species. The specimens of *Mesobiotus ethiopicus* sp. nov. are most similar to two taxa of the *harmsworthi* complex: *M. harmsworthi obscurus* (Dastych, 1985) and *M. peterseni* (Maucci, 1991). Nevertheless, the new species can be easily distinguished from the first of these taxa by the absence of additional teeth in the oral cavity and by a different accessory point morphology on the claws, and it can be distinguished from the second species mostly by a completely different egg process anatomy. *Mesobiotus ethiopicus* sp. nov. is only the second tardigrade species formally described as new for science from Ethiopia so far.

Key words: Africa, 18S rRNA, 28S rRNA, COI, Ethiopia, integrative taxonomy

1. Introduction

The Federal Democratic Republic of Ethiopia (Ethiopia) is a country located on the northeast African peninsula (the so-called Horn of Africa). It does not have access to the sea and is surrounded by six countries; from the north in a clockwise direction, they are as follows: Eritrea, Djibouti, Somalia, Kenya, South Sudan, and Sudan. The wide spectrum of climate, soils, natural vegetation (Friis et al., 2010), and settlement patterns is related to the extreme diversity of terrain (highland complex of mountains, plateaus dissected by the Great Rift Valley, lowlands, steppes, semideserts). The wide range of elevation in this region has resulted in ecological differentiation, creating diverse habitats from deserts to tropical forests. Such ecological variation may have played a considerable role in some biological processes, such as the ecological isolation of populations and evolution of endemic species. Although the large African vertebrates are well known, there are many groups of invertebrates that are poorly investigated (Myers et al., 2000). One such group is the phylum Tardigrada, which consists of microscopic animals inhabiting terrestrial, freshwater, and marine environments throughout the world (e.g., Nelson and Marley, 2000; Nelson et al., 2015). Currently the number

of known species is over 1200 (Degma et al., 2016); each year, several new taxa are described.

To date, only eight tardigrade species have been found in Ethiopia (McInnes et al., 2017), and all of them were reported in one faunistic study in the Oromia Region by Pardi (1941). Only one of them, *Minibiotus granatai* (Pardi, 1941), was formally described as a species new to science specifically from this country, and it has not been found in any other locality throughout the world. The other species reported from Ethiopia by Pardi (1941) are as follows: *Isohypsibius nodosus* (Murray, 1907a), *Pseudechiniscus suillus* (Ehrenberg, 1853) sensu lato, *Milnesium tardigradum* Doyère, 1840 s.l., *Macrobiotus hufelandi* C.A.S. Schultze, 1833 s.l., *Mesobiotus harmsworthi* (Murray, 1907b) s.l., *Minibiotus intermedius* (Plate, 1888) s.l., and *Paramacrobiotus richtersi* (Murray, 1911) s.l. The last six of these are representative taxa for species complexes that have been described from the Palearctic region; thus, these records should be treated with extreme caution. *Isohypsibius nodosus* (Murray, 1907a) was described from the Republic of South Africa (Cape Province), and it has also been reported from several additional localities in different parts of the world (see McInnes, 1994; McInnes et al., 2017), suggesting a

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putative species complex. Interestingly, Murray (1913) and Pardi (1941) underlined some minute differences in the size of nodules in African specimens from Kenya and Ethiopia, respectively. In consequence, this record should be treated as dubious.

Recently, some species complexes that include phenotypically similar species have been separated into multiple genera by combining morphological and molecular analyses (Vecchi et al., 2016). One example is the genus *Mesobiotus* Vecchi et al., 2016, which was erected from the genus *Macrobiotus* C.A.S. Schultze, 1834. It is composed of species from two former informal taxonomic groups: the *Macrobiotus harmsworthi* group and the *Macrobiotus furciger* group. Species that belong to the genus *Mesobiotus* are characterized by Y-shaped double claws with evident septum, cuticle without pores, three roundish macropylacoids, and one micropylacoid that is situated closely (less than its length) to the third macropylacoid, and by freely laid eggs with conical to hemispherical processes.

With an integrative taxonomy approach applying detailed morphological (phase contrast microscopy [PCM], scanning electron microscopy [SEM]), morphometric (PCM), and molecular (18S rRNA, 28S rRNA, and COI markers) analyses, we were able to assemble comprehensive data for *Mesobiotus ethiopicus* sp. nov. collected in Ethiopia, allowing us to describe it as a species new to science, and to differentiate it from others within the current *Mesobiotus harmsworthi* group.

2. Materials and methods

2.1. Sample processing

The moss sample from shaded rocks was collected by Asger Ken Pedersen on 15 February 2016 in open Afro-alpine hilly terrain from Amhara Regional State, in the Semien Mountains (Ethiopia). The sample was collected and examined for terrestrial tardigrades using standard methods (e.g., Stec et al., 2015). A total of 22 individuals and 16 eggs of the new species were extracted from the sample, and split into three groups: 19 animals and 13 eggs were mounted on microscope slides in Hoyer's medium, three eggs were prepared for imaging with scanning electron microscopy (SEM), three specimens were used for DNA sequencing.

Apart from the new species, the sample also contained two specimens of *Pseudechiniscus* sp., one specimen of *Isohypsibius* sp., one specimen of *Ramazzottius* sp., three specimens of *Macrobiotus hufelandi* group species, and 24 specimens of *Testechiniscus* sp.

2.2. Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides according to Morek et al. (2016) in a small drop of Hoyer's medium, and secured with a

cover slip. The slides were then dried for 2 weeks at room temperature and then for 5 days at 60 °C. Dried slides were sealed with transparent nail polish and examined with a Nikon Eclipse 50i phase contrast light microscope (PCM) equipped with a Nikon Digital Sight DS-L2 digital camera. Eggs for SEM were processed as follows: they were first subjected to water/ethanol and an ethanol/acetone series, and then to CO₂ critical point drying in a critical point dryer (Autosamdri-815 series A, Tousimis Research Corporation, Rockville, MD, USA). Specimens were then coated with platinum–palladium using a sputter coater (JEOL JFC-2300HR high resolution fine coater). Finally, specimens were examined under high vacuum in a JEOL Field Emission Scanning Electron Microscope JSM6335F (Jeol, Tokyo, Japan) located in the Natural History Museum of Denmark, University of Copenhagen, in the Zoological Museum.

All figures were assembled in Corel Photo-Paint X6, v.16.4.1.1281. For deep structures that could not be fully focused on in a single photograph, a series of 2–10 images were taken every c. 0.25 µm and then assembled into a single deep-focus image.

2.3. Morphometrics and morphological nomenclature

Sample size for morphometrics was chosen following recommendations by Stec et al. (2016). All measurements are given in micrometres (µm). Structures were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The terminology for the buccal apparatus and claw types follows Pilato and Binda (2010). The terminology used to describe the oral cavity armature follows that established by Hansen and Katholm (2003), Michalczyk and Kaczmarek (2003) and updated by Hansen et al. 2017. Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). Buccal tube width was measured as the external and internal diameter at the level of the stylet support insertion point. Macropylacoid length sequence is given according to Kaczmarek et al. (2014). Lengths of the claw branches were measured from the base of the claw (i.e. excluding the lunula) to the top of the branch, including accessory points. The *pt* index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato, 1981). Distance between egg processes was measured as the shortest line connecting base edges of the two closest processes. Morphometric data were handled using the “Parachela” v.1.2 template available from the Tardigrada Register (Michalczyk and Kaczmarek, 2013). Tardigrade taxonomy follows Bertolani et al. (2014). All raw data underlying the description of *Mesobiotus ethiopicus* sp. nov. are deposited in the Tardigrada Register (Michalczyk and Kaczmarek, 2013) under www.tardigrada.net/register/0045.htm.

2.4. Genotyping

For DNA sequencing, three paragenophores (sensu Pleijel et al., 2008) were used. The DNA was extracted from individual animals following a Chelex 100 resin (Bio-Rad) extraction method by Casquet et al. (2012), with modifications described in detail in Stec et al. (2015). We sequenced three DNA fragments (two nuclear: nDNA; and one mitochondrial: mtDNA) differing in mutation rates (from the most to the least conservative): the small ribosome subunit (18S rRNA, nDNA), the large ribosome subunit (28S rRNA, nDNA), and the cytochrome oxidase subunit I (COI, mtDNA). All fragments were amplified and sequenced according to the protocols described in Stec et al. (2015); primers and original references for specific PCR programs are listed in Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit v.7.2.5 (Hall, 1999) and submitted to GenBank. The accession numbers for each 18S rRNA, 28S rRNA, and COI are: MF678793, MF678792, and MF678794, respectively.

Because of the scarcity of available DNA data for the *harmsworthi* group species, all *Mesobiotus* sequences currently deposited in GenBank were used for the genotypic differential diagnosis:

COI: *M. philippinicus* (KX129796 by Mapalo et al., 2016), *M. hilariae* (KT226108 by Vecchi et al., 2016);

28S rRNA: *M. philippinicus* (KX129794 by Mapalo et al., 2016) (only one sequence was used since the other published 28S rRNA fragments correspond to a different region of the gene);

18S rRNA: *M. harmsworthi* (HQ604967–70 by Bertolani et al., 2014; KT226073–4 by Vecchi et al., 2016), *M. cf. mottai* (KT226072 by Vecchi et al., 2016), *M. hilariae* (KT226068–71 by Vecchi et al., 2016), *M. polaris* (KT226075–8 by Vecchi et al., 2016), *M. philippinicus* (KX129793 by Mapalo et al., 2016).

The sequences were aligned with the ClustalW Multiple Alignment tool (Thompson et al., 1994) implemented in BioEdit. The aligned sequences were then trimmed to 741 bp (18S rRNA), 729 bp (28S rRNA), and 638 bp (COI). MEGA v.6.0 (Tamura et al., 2013) was used for computing uncorrected genetic pairwise distances calculations and for the COI sequences translation to polypeptides to test against pseudogenes.

3. Results

3.1. Taxonomic account of the new species

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980

Superfamily: Macrobiotidea Thulin, 1928 (in Marley et al., 2011)

Family: Macrobiotidae Thulin, 1928

Genus: *Mesobiotus* Vecchi, Cesari, Bertolani, Jönsson, Rebecchi, and Guidetti, 2016

***Mesobiotus ethiopicus* sp. nov.**

(Tables 2–3; Figures 1–6)

3.2. Material examined: 19 animals (including one simplex), 13 eggs mounted on microscope slides in Hoyer's medium (some of the eggs were embrionated), three eggs fixed on SEM stubs, and three specimens processed for DNA sequencing.

3.3. Type locality: 13°16'N, 38°12'E; 3750 m a.s.l.: Ethiopia, Amhara Regional State, shaded rocks in the Semien Mountains, open Afro-alpine terrain, moss from a rock, coll. 15 February 2016 by Asger Ken Pedersen.

3.4. Etymology: The species is named after the country where it was discovered.

3.5. Type depositories: Holotype: slide ET.004.17, 13 paratypes (slides: ET.004/*, where the asterisk can be substituted by any of the following numbers: 17–19) and 8 eggs (slide: ET.004.20) are deposited at the Department

Table 1. Primers used for sequencing of 18S rRNA, 28S rRNA, and COI genes of *Mesobiotus ethiopicus* sp. nov.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR program
18S rRNA	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands et al. (2008)	Zeller (2010)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTACCTAC	Sands et al. (2008)	
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov et al. (2012)	Mironov et al. (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)	
COI	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	Michalczyk et al. (2012)
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)	

Table 2. Measurements [in μm] of selected morphological structures of individuals of *Mesobiotus ethiopicus* sp. nov. from Ethiopia mounted in Hoyer's medium. N – number of specimens/structures measured; Range refers to the smallest and the largest structure among all measured specimens; SD – standard deviation.

Character	N	Range		Mean		SD		Holotype	
		μm	pt	μm	pt	μm	pt	μm	pt
Body length	15	316	– 511	–	402	63	–	501	–
Buccopharyngeal tube									
Buccal tube length	15	37.2	– 58.9	–	46.9	–	6.6	58.9	–
Stylet support insertion point	15	27.8	– 44.2	74.5	– 76.6	35.2	75.0	5.1	0.5
Buccal tube external width	14	4.5	– 7.6	11.1	– 14.2	6.1	12.8	1.0	1.0
Buccal tube internal width	14	2.9	– 5.5	7.8	– 10.5	4.3	9.1	0.9	0.9
Ventral lamina length	14	19.8	– 33.1	53.2	– 60.9	27.3	57.7	4.1	2.2
Placoid lengths									
Macroplacoid 1	15	6.0	– 9.6	14.3	– 20.4	7.8	16.6	1.5	1.5
Macroplacoid 2	15	4.6	– 8.1	11.0	– 16.1	6.1	12.9	1.2	1.5
Macroplacoid 3	15	5.3	– 9.2	12.4	– 19.1	7.1	15.1	1.6	1.8
Microplacoid	15	2.1	– 5.3	4.8	– 10.3	3.2	6.8	1.0	1.7
Macroplacoid row	15	18.0	– 31.5	45.8	– 62.0	24.3	51.4	4.7	4.5
Placoid row	15	21.8	– 36.0	54.0	– 72.6	28.4	60.4	5.3	5.1
Claw 1 lengths									
External primary branch	14	8.7	– 14.2	20.1	– 28.9	11.2	23.6	1.9	2.1
External secondary branch	13	6.9	– 11.8	15.3	– 24.8	9.0	19.0	1.7	2.6
Internal primary branch	11	7.0	– 14.2	16.4	– 24.2	9.7	20.4	2.1	2.4
Internal secondary branch	6	6.5	– 10.8	16.6	– 20.2	8.9	18.0	1.8	1.2
Claw 2 lengths									
External primary branch	14	8.8	– 15.8	20.6	– 29.5	11.8	25.4	2.1	2.5
External secondary branch	12	6.9	– 12.1	16.1	– 24.6	10.0	21.0	1.7	2.7
Internal primary branch	8	7.7	– 11.7	17.8	– 22.7	9.5	20.7	1.5	2.0
Internal secondary branch	5	6.7	– 9.0	13.8	– 22.1	8.1	18.1	1.0	3.8
Claw 3 lengths									
External primary branch	13	9.4	– 14.8	22.4	– 29.5	11.9	25.5	2.0	2.2
External secondary branch	11	7.1	– 12.5	16.6	– 23.9	9.7	20.4	1.9	2.7
Internal primary branch	9	7.1	– 12.0	16.6	– 23.7	9.4	21.0	1.7	1.9
Internal secondary branch	7	6.6	– 10.9	15.4	– 21.2	8.9	19.4	1.8	2.1
Claw 4 lengths									
Anterior primary branch	13	9.1	– 16.3	21.3	– 28.7	11.7	24.8	2.4	2.6
Anterior secondary branch	8	7.3	– 11.7	17.1	– 22.7	9.5	20.6	1.8	1.9
Posterior primary branch	13	9.7	– 15.1	22.7	– 30.2	12.6	26.8	2.0	2.3
Posterior secondary branch	11	7.5	– 11.8	19.2	– 23.2	9.9	21.1	1.4	1.3

Table 3. Measurements (in μm) of selected morphological structures of eggs of *Mesobiotus ethiopicus* sp. nov. mounted in Hoyer's medium (N-number of eggs/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD-standard deviation).

Character	N	Range	Mean	SD
Egg bare diameter	12	62.2 – 74.6	68.8	3.7
Egg full diameter	12	89.5 – 117.5	104.1	7.2
Process height	33	15.3 – 23.6	19.7	2.4
Process base width	33	13.1 – 22.5	18.2	1.7
Process base/height ratio	33	77% – 117%	94%	9%
Distance between processes	33	2.4 – 5.1	4.1	0.7
Number of processes on the egg circumference	10	10 – 12	10.7	0.7

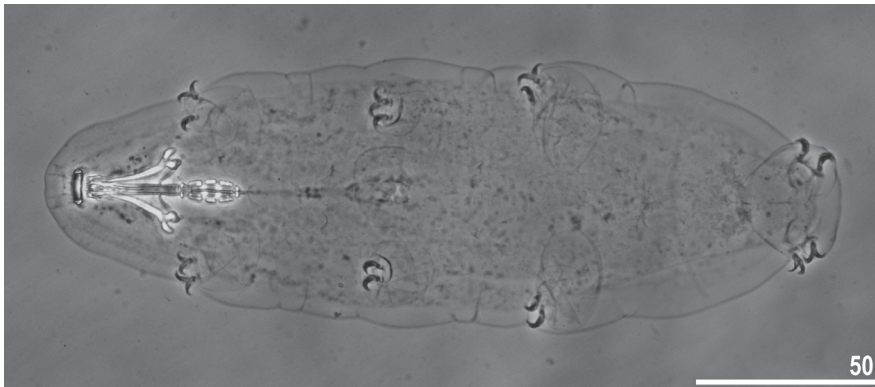


Figure 1. *Mesobiotus ethiopicus* sp. nov. – PCM image of habitus: A – dorsoventral projection (holotype). Scale bar in μm .

of Entomology, Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387, Kraków, Poland. Five paratypes (slide: ET.004.21) and five eggs (slide: ET.004.22) are deposited in the Zoological Museum, Natural History Museum of Denmark, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark.

3.6. Description of the new species

Animals (measurements and statistics in Table 2): In live animals, body almost transparent in young specimens and white in adults; after fixation in Hoyer's medium, body transparent (Figure 1A). Eyes absent (before and after fixation). Body cuticle smooth, i.e. without pores, spines, or sculpturing. Granulation on all legs absent.

Mouth anteroventral. Buccopharyngeal apparatus of the *Macrobiotus* type, with the ventral lamina and 10 small peribuccal lamellae (Figure 2A). The oral cavity armature well developed and composed of three bands of teeth (Figures 2B–2G). The first band of teeth is composed of numerous small granules arranged in several rows situated anteriorly in the oral cavity, just behind the bases of the peribuccal lamellae (Figures 2B–2G). The second band of

teeth is situated between the ring fold and the third band of teeth and is composed of ridges parallel to the main axis of the buccal tube and granules, larger than those in the first band (Figures 2B–2G). Some teeth in the second band are clearly larger than other teeth in this band (Figures 2B–2G, flat arrowhead). The teeth of the third band are located within the posterior portion of the oral cavity, between the second band of teeth and the buccal tube opening (Figures 2B–2G). The third band of teeth is divided into the dorsal and the ventral portion. Under PCM, both dorsal and ventral teeth are visible as two lateral and one median transverse ridges (Figures 2B–2G). The ventromedian tooth is roundish and sometimes constricted or fully divided into two separate roundish teeth (Figures 2C, 2E, 2G, arrow). Pharyngeal bulb ovoid (Figures 2A), with triangular apophyses, three rod-shaped macroplacoids and the drop-shaped (in lateral view) or triangular (in dorsoventral view) microplacoid placed closely to the third macroplacoid (Figures 2A, 2H, and 2I). The macroplacoid length sequence is $2 < 3 < 1$. The first macroplacoid is anteriorly narrowed and the third has a subterminal constriction (Figures 2H and 2I, empty arrowhead).

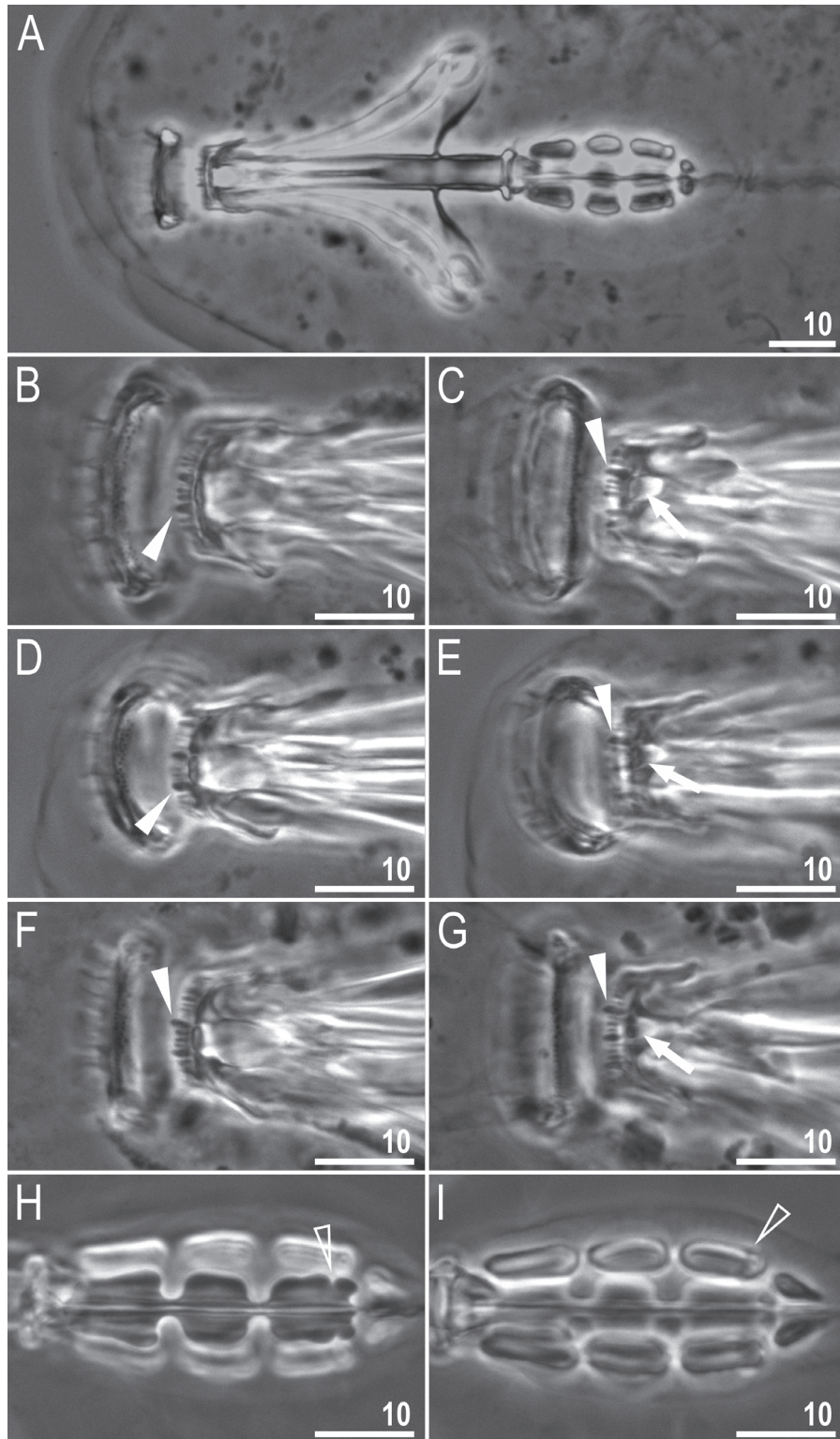


Figure 2. *Mesobiotus ethiopicus* sp. nov. – PCM images of the buccal apparatus: A – an entire buccal apparatus (paratype); B–C – the oral cavity armature of the holotype, dorsal and ventral teeth respectively; D–E – the oral cavity armature of the paratype, dorsal and ventral teeth respectively; F–G – the oral cavity armature of the paratype, dorsal and ventral teeth respectively; H–I – placoid morphology of the paratype, ventral and dorsal placoids, respectively. Filled flat arrowheads indicate the larger teeth in the second band of teeth, arrows indicate the medioventral tooth in the third band of teeth, empty flat arrowheads indicate subterminal constrictions in the third macroplacoid. Scale bars in µm.

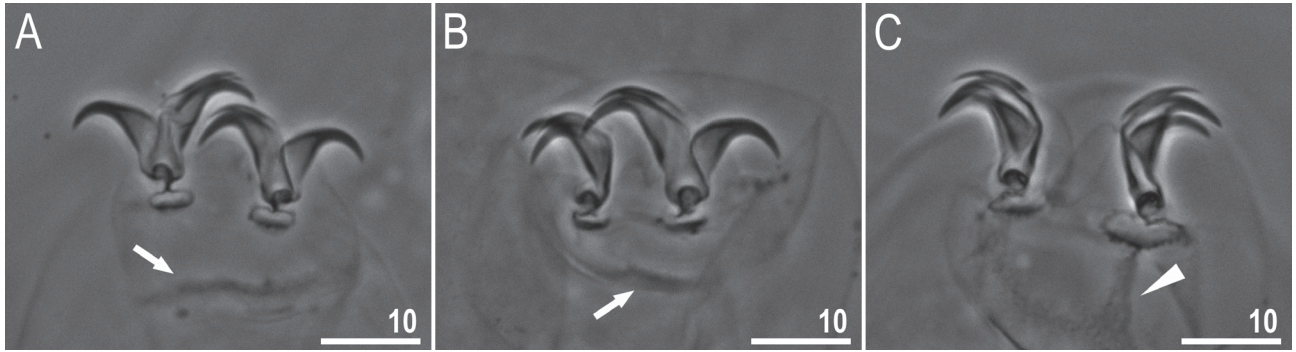


Figure 3. *Mesobiotus ethiopicus* sp. nov. – PCM images of claws: A – claws I with smooth lunules; B – claws II with smooth lunules; C – claws IV with moderately serrated lunules. All claw photos from the holotype. Arrows indicate the cuticular bars, the filled arrowhead indicates the horseshoe structure connecting the anterior and the posterior claw. Scale bars in μm .

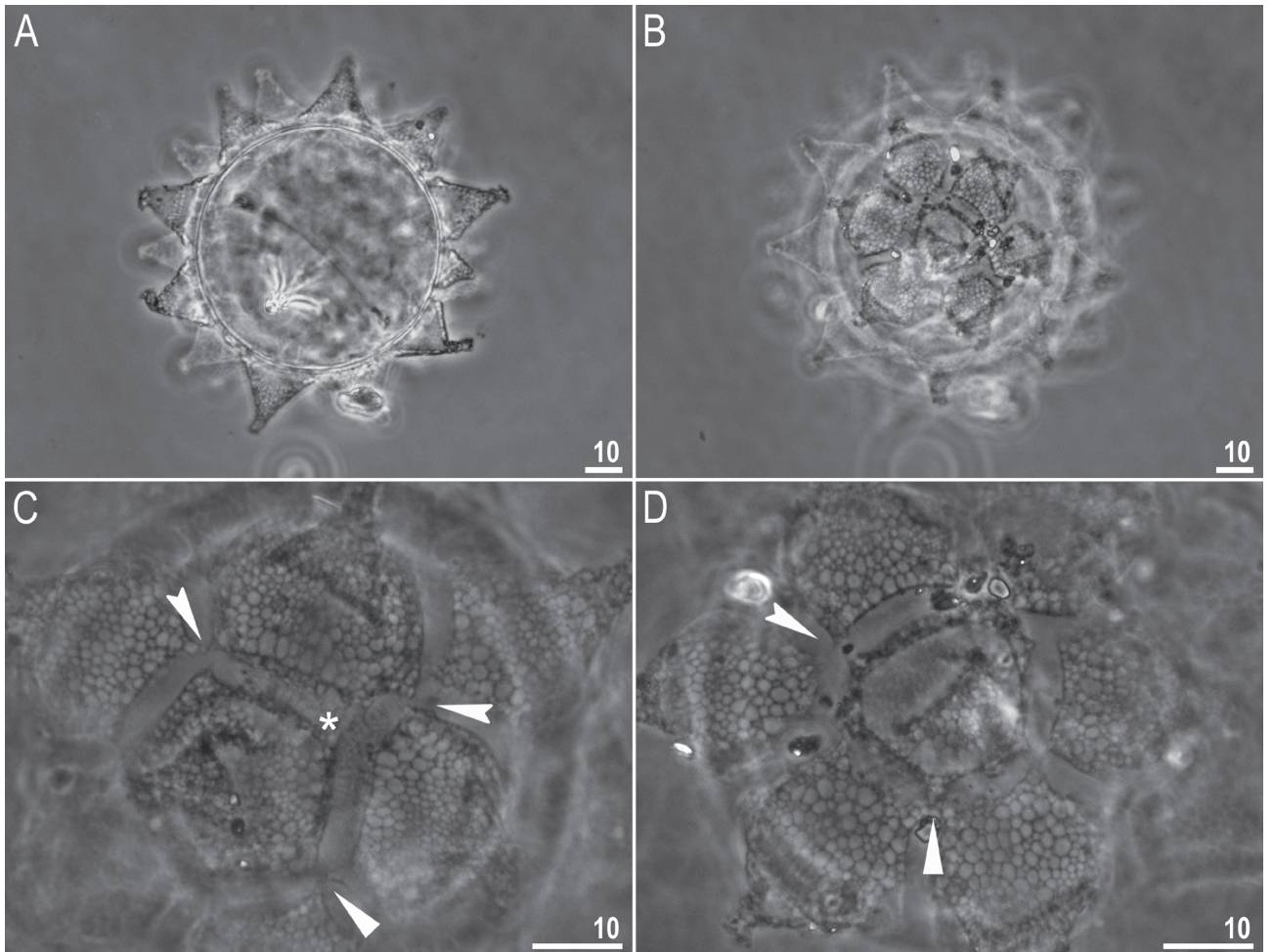


Figure 4. *Mesobiotus ethiopicus* sp. nov. – PCM images of the eggs: A – midsection; B – surface; C–D – surfaces under 1000 \times magnification. Asterisk indicates the wrinkled surface inside the areolae, filled indented arrowheads indicate poorly developed connection between the processes, filled flat arrowheads indicate rare fully developed connections between processes. Scale bars in μm .

Claws of the *Mesobiotus* type, with a peduncle connecting the claw to the lunula, a basal septum, and well-developed accessory points situated parallel to the primary

branch (Figures 3A–3C). Lunules under claws I–III smooth (Figures 3A, 3B), but under claws IV slightly serrated (Figure 3C). Single transverse bars present beneath claws

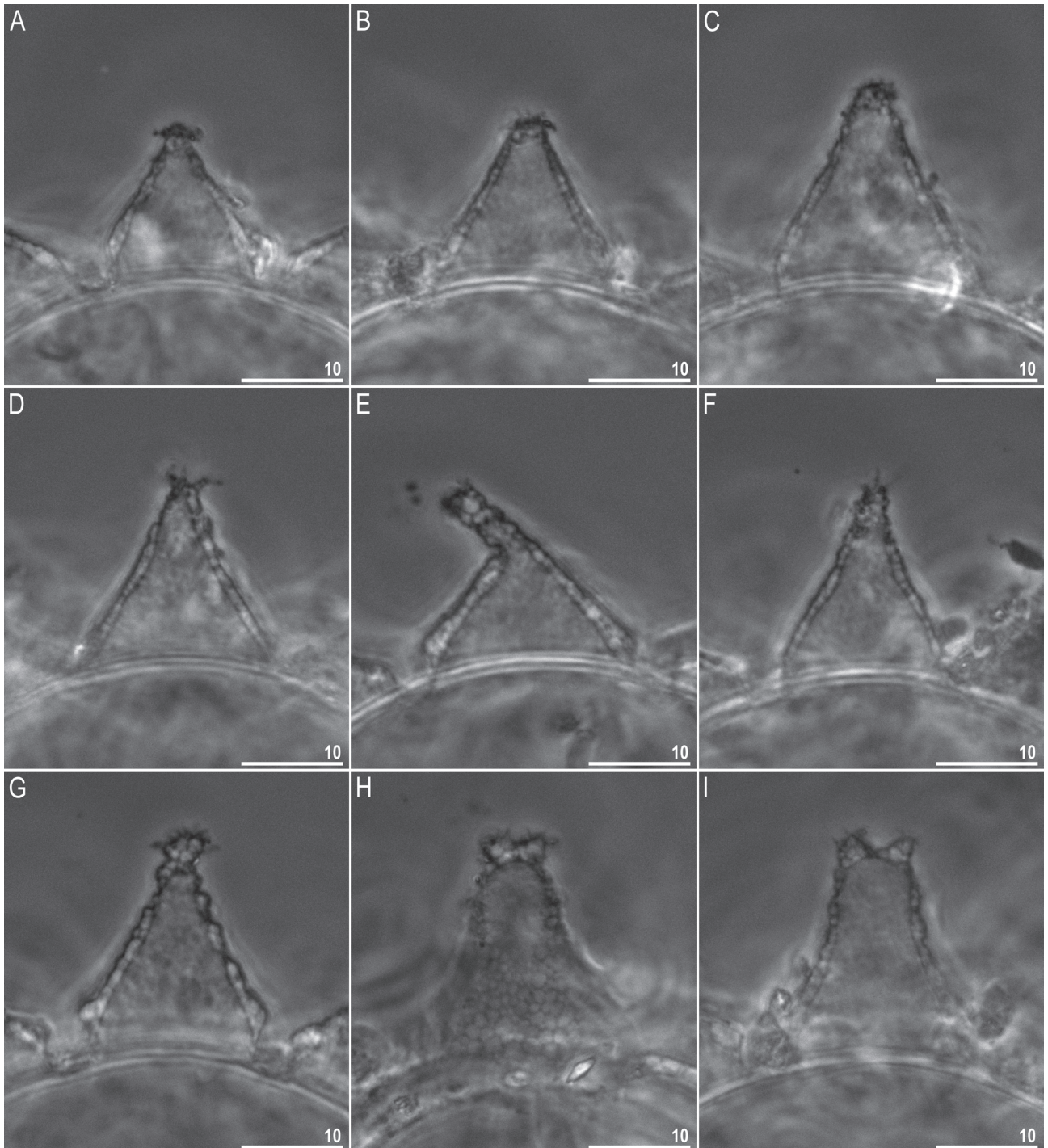


Figure 5. *Mesobiotus ethiopicus* sp. nov. – PCM images of the midsection of various types of egg processes. Scale bars in μm .

I–III (Figures 3A, 3B, arrow), whereas a horseshoe-shaped structure connects the anterior and posterior lunules on claws IV (Figure 3C, filled flat arrowhead).

Eggs (measurements and statistics in Table 3): Laid freely, white, spherical with conical processes (Figures 4A and 6A, 6B). The processes are equidistant from each other

(Figures 4A–4D and 6A, 6B) with bases of hexagonal shape (Figures 4B–4D and 6A–6D). The process surface seems to be reticulated under PCM but smooth under SEM (Figures 4A–4D, 5A–5I, and 6A–6H). The labyrinthine layer within the process walls appears as reticulation under PCM, with meshes varying in diameter on each process, with

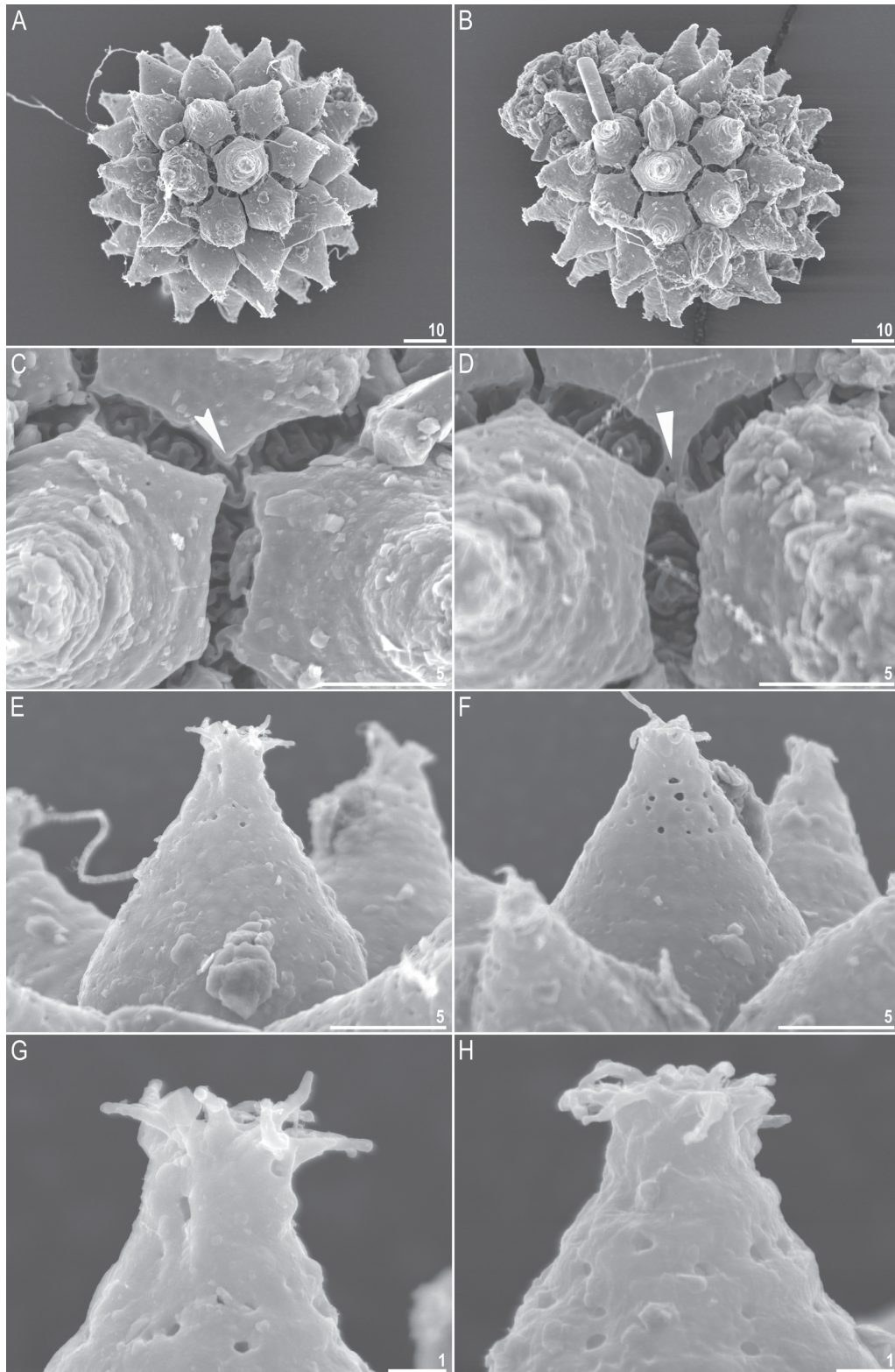


Figure 6. *Mesobiotus ethiopicus* sp. nov. – SEM images of eggs: A–B – entire view of 2 different eggs; C–D – egg surface between processes; E–F – egg processes; G–H – a top part of the processes terminated by several short flexible filaments. Filled indented arrowheads indicate poorly developed connection between the processes, filled flat arrowheads indicate rare fully developed connections between processes. Scale bars in µm.

decreasing mesh size from bottom to top on each process (Figures 4C, 4D and 5H). Several pores in the top portion of the processes are present in the external process walls (clearly visible only in SEM) (Figures 6E–6H). Processes are terminated by several short, thin, and flexible filaments very susceptible to fracture, which are visible in both PCM (Figures 5A–5I) and SEM (Figures 6E–6H). Moreover, the processes are sometimes bifurcated (Figures 5H–5I). Six flat, narrow, often not fully developed (Figures 4C, 4D and 6C, filled indented arrowhead), and only sometimes connected (Figures 4C, 4D and 6D, filled flat arrowhead) areoles are present around each process. The inner areole surface is wrinkled but this trait is only clearly visible in SEM (Figures 6C, 6D), rarely and barely visible in PCM (Figure 4C, asterisk).

3.7. DNA sequences and p-distances comparisons

We obtained sequences for all three of the above mentioned molecular markers from all three paragenophores. All markers were represented by single private haplotypes:

The 18S rRNA sequence (GenBank: MF678793), 800 bp long;

The 28S rRNA sequence (GenBank: MF678792), 772 bp long;

The COI sequence (GenBank: MF678794), 638 bp long.

The ranges of uncorrected genetic p-distances between the new species and species of the *Mesobiotus harmsworthi* group, for which sequences are available from GenBank, are as follows (from the most to the least conservative):

18S rRNA: 1.1%–5.7%, with the most similar being *M. philippinicus* from the Philippines (KX129793) and the least similar being *M. cf. mottai* from the Antarctic (KT226072);

28S rRNA: 7.2% between the new species and *M. philippinicus* from the Philippines (KX129794);

COI: 24.0%–24.3%, with the most similar being *M. philippinicus* from the Philippines (KX129796) and the least similar *M. hilariae* from Antarctica (KT226108).

4. Discussion

4.1. Phenotypic differential diagnosis

Having three rod-shaped macroplacoids and a relatively large microplacoid placed close to the third macroplacoid places *Mesobiotus ethiopicus* sp. nov. in the genus *Mesobiotus*. These morphological criteria for the animals, together with conical egg processes, places the new species within the *harmsworthi* group (Kaczmarek et al., 2011). By having a completely smooth cuticle, even on the legs, and eggs without fully developed areoles, the new species is similar to two species of the *harmsworthi* group but differs specifically from:

- *M. harmsworthi obscurus* (Dastych, 1985) known only from the locus typicus and Ural Mountains (Perm

district, Russia) (Biserov, 1991), by: absence of eyes (eyes present in *M. harmsworthi obscurus*), absence of additional teeth in the oral cavity (a few to a dozen additional teeth in the oral cavity present in *M. harmsworthi obscurus* situated between second and third band of teeth), presence of several evidently larger teeth in the second band of teeth in the oral cavity armature (second band of teeth uniform in *M. harmsworthi obscurus*), a different morphology of accessory points on the primary branches of all the claws (typically developed accessory points orientated parallel to the primary branches in the new species vs. strikingly large and upward pointing accessory points in *M. harmsworthi obscurus*), different morphology of egg process endings (processes terminated by several short, thin, and flexible filaments susceptible to fracture in the new species vs. absence of flexible filaments at the process ends in *M. harmsworthi obscurus*).

- *M. peterseni* (Maucci, 1991) known only from the locus typicus in Greenland, by: presence of several evidently larger teeth in the second band of teeth in the oral cavity armature (second band of teeth uniform in *M. peterseni*), different macroplacoid sequence ($2 < 3 < 1$ in the new species vs. $2 < 1 < 3$ in *M. peterseni*), different morphology of the egg processes (conical processes with flexible filaments at the endings and with differentiated reticulation on process surface in the new species vs. dome-shaped process without flexible filaments at the endings, with uniformly reticulated processes wall surface and differentiated reticulation only on the process top, which resembles one large pore surrounded by several smaller pores in *M. peterseni*).

4.2. Comments on molecular results

The comparison between sequences obtained in our study and sequences deposited in GenBank showed that our DNA sequences are clearly different and unique. This result support our hypothesis about new species erection. Nevertheless, our analysis is based only on a very limited set of molecular data currently available in a public database. It stresses that effort should be made to cover this gap in the molecular data for the genus *Mesobiotus*. It will be especially important not only for species identification and delineation, but also to infer more precisely the phylogenetic relationships within this taxon.

Mesobiotus ethiopicus sp. nov. is the second tardigrade species formally described from Ethiopia as a taxon new to science. The new species erection and delineation was made possible by using an integrative approach including scanning electron microscopy (SEM), phase contrast microscopy (PCM), and DNA sequencing. Despite the scarcity of molecular data for the genus *Mesobiotus* and the lack of calculated DNA distance thresholds for species delineation within the phylum Tardigrada (Michalczyk

et al., 2012), the differences in uncorrected p-distances, together with clear morphological differences shown in our work, unambiguously support the erection of the new species. As of now, only nine tardigrade taxa have been reported from Ethiopia, but past records of all species, excluding *Mesobiotus ethiopicus* sp. nov. and *Minibiotus granatai* (Pardi, 1941), should be treated with great caution since they are nominal taxa for species complexes for which the descriptions are imprecise (Kaczmarek et al., 2015).

Nomenclatural acts

This work and the nomenclatural acts it contains have been registered in ZooBank. The ZooBank Life Science Identifier (LSID) for this publication is: <http://zoobank.org/urn:lsid:zoobank.org:pub:395FA2EC-2295-4A14-B4F2-A0880010F692>.

References

- Bertolani R, Guidetti R, Marchioro T, Altiero T, Rebecchi L, Cesari M (2014). Phylogeny of Eutardigrada: new molecular data and their morphological support lead to the identification of new evolutionary lineages. *Mol Phylogenet Evol* 76: 110-126.
- Biserov VI (1991). An annotated list of Tardigrada from European Russia. *Zool. Jahrb (Syst Økol)* 118: 193-216.
- Casquet J, Thebaud C, Gillespie RG (2012). Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Mol Ecol Resour* 12: 136-141.
- Dastyh H (1985). West Spitsbergen Tardigrada. *Acta Zool Cracov* 28: 169-214.
- Degma P, Bertolani R, Guidetti R (2016). Actual checklist of Tardigrada species (2009–2016, Version 30: 15-09-2016). (<http://www.tardigrada.modena.unimo.it/miscellanea/Actual%20checklist%20of%20Tardigrada.pdf>).
- Doyère LMF (1840). Memoire sur les Tardigrades. I. *Annales des Sciences Naturelles, Paris, Series 2*, 14: 269-362.
- Ehrenberg CG (1853). Diagnoses novarum formarum. *Verh. K. Ak. Wiss. Berlin* 8: 526-533.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3: 294-299.
- Friis I, Demissew S, Van Beugel P (2010). Atlas of the Potential Vegetation of Ethiopia. *Biol Skr* 58: 1-307.
- Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.
- Hansen JG, Katholm AK (2003). A study of the genus *Amphibolus* from Disko Island with special attention on the life cycle of *Amphibolus nebulosus* (Eutardigrada, Eohypsibiidae). *Arctic Biology Field Course - Qeqertarsuaq*, 2002. København: H.C.Ø.-Tryk 2003: 129-163.
- Hansen JG, Kristensen RM, Bertolani R, Guidetti R (2017). Comparative analyses of Bertolanius species (Eohypsibiidae; Eutardigrada) with the description of Bertolanius birnae sp. nov from northern polar regions. *Polar Biol* 40:123-140.
- Kaczmarek Ł, Cytan J, Zawierucha K, Diduszko D, Michalczyk Ł (2014). Tardigrades from Peru (South America), with descriptions of three new species of Parachela. *Zootaxa* 3790(2): 357-379.
- Kaczmarek Ł, Goldyn B, Prokop ZM, Michalczyk Ł (2011). New records of Tardigrada from Bulgaria with the description of *Macrobiotus binieki* sp. nov. (Eutardigrada: Macrobiotidae) and a key to the species of the *harmsworthi* group. *Zootaxa* 2781: 29-39.
- Kaczmarek Ł, Michalczyk Ł, McInnes SJ (2015). Annotated zoogeography of non-marine Tardigrada. Part II: South America. *Zootaxa* 3923: 1-107.
- Mapalo M, Stec D, Mirano-Bascos D, Michalczyk Ł (2016). *Mesobiotus philippinicus* sp. nov., the first limnoterrestrial tardigrade from the Philippines. *Zootaxa* 4126: 411-426.
- Marley NJ, McInnes SJ, Sands CJ (2011). Phylum Tardigrada: A re-evaluation of the Parachela. *Zootaxa* 2819: 51-64.
- Maucci W (1991). Tre nuove specie di Eutardigradi della Grenlandia Meridionale. *Bol Mus civ St nat Verona* 15: 279-289.
- McInnes SJ (1994). Zoogeographic distribution of terrestrial/freshwater tardigrades from current literature. *J Nat Hist* 28: 257-352.

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- McInnes SJ, Kaczmarek Ł, Michalczyk Ł (2017). Annotated zoogeography of non-marine Tardigrada. Part IV: Africa. Zootaxa.
- Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000). Biodiversity hotspots for conservation priorities. Nature 403: 853-858.
- Michalczyk Ł, Kaczmarek Ł (2003). A description of the new tardigrade *Macrobiotus reinhardti* (Eutardigrada, Macrobiotidae, *harmsworthi* group) with some remarks on the oral cavity armature within the genus *Macrobiotus* Schultze. Zootaxa 331: 1-24.
- Michalczyk Ł, Kaczmarek Ł (2013). The Tardigrada Register: a comprehensive online data repository for tardigrade taxonomy. J Limnol 72: 175-181.
- Michalczyk Ł, Welnicz W, Frohme M, Kaczmarek Ł (2012). Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. Zootaxa 3154: 1-20.
- Mironov SV, Dabert J, Dabert M (2012). A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the Long-tailed Tit *Aegithalos caudatus* (Passeriformes: Aegithalidae): morphological description with DNA barcode data. Zootaxa 3253: 54-61.
- Morek W, Stec D, Gąsiorek P, Schill RO, Kaczmarek Ł, Michalczyk Ł (2016). An experimental test of eutardigrade preparation methods for light microscopy. Zool J Linn Soc-Lond 178: 785-793.
- Murray J (1907a). Some South African Tardigrada. J R Micr Soc London 5: 515-524.
- Murray J (1907b). Arctic Tardigrada, collected by Wm. S. Bruce. Trans R Soc Edinburgh 45: 669-681.
- Murray J (1913). African Tardigrada. J R Micr Soc London 2: 136-144.
- Nelson DR, Guidetti R, Rebecchi L (2015). Phylum Tardigrada. In: Thorp J, Rogers DC. Ecology and General Biology: Vol. 1. Thorp and Covich's Freshwater Invertebrates (4th Revised Edition). Amsterdam, Netherlands: Academic Press Inc., pp. 347-380.
- Nelson DR, Marley NJ (2000). The biology and ecology of lotic Tardigrada. Fresh Biol 44: 93-108.
- Pardi L (1941). Tardigrada. Miss. Biol. Sagan-Omo Roma Zool 6: 221-232.
- Pilato G (1981). Analisi di nuovi caratteri nello studio degli Eutardigrada. Animalia 8: 51-57.
- Pilato G, Binda MG (2010). Description of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification. Zootaxa 2404: 1-54.
- Pleijel F, Jondelius U, Norlinder E, Nygren A, Oxelman B, Schander C, Sundberg P, Thollesson M (2008). Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. Mol Phylogenet Evol 48: 369-371.
- Richters F (1926). Tardigrada. In: Kükenthal W, Krumbach T, editors. Handbuch der Zoologie: Volume 3. Berlin, Germany: Walter de Gruyter & Co, p. 58-61.
- Sands C J, McInnes SJ, Marley NJ, Goodall-Copestake W, Convey P, Linse K (2008). Phylum Tardigrada: an "individual" approach. Cladistics 24: 1-18.
- Schultze CAS (1834). *Macrobiotus Hufelandii* animal e crustaceorum classe novum, reviviscendi post diuturnam asphixiam et ariditatem potens, etc. 8, 1 tab. Berlin, Germany: C. Curths.
- Schuster RO, Nelson DR, Grigarick AA, Christenberry D (1980). Systematic criteria of the Eutardigrada. Trans Am Microsc Soc 99: 284-303.
- Stec D, Gąsiorek P, Morek W, Kosztyla P, Zawierucha K, Michno K, Kaczmarek Ł, Prokop ZM, Michalczyk Ł (2016). Estimating optimal sample size for tardigrade morphometry. Zool J Linn Soc-Lond 178: 776-784.
- Stec D, Smolak R, Kaczmarek Ł, Michalczyk Ł (2015). An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. Zootaxa 4052: 501-526.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30: 2725-2729.
- Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.
- Thulin G (1928). Über die Phylogenie und das System der Tardigraden. Hereditas 11: 207-266.
- Vecchi M, Cesari M, Bertolani R, Jönsson IK and Guidetti R (2016). Integrative systematic studies on tardigrades from Antarctica identify new genera and new species within Macrobiotidea and Echiniscoidea. Invertebr Syst 30: 303-322.
- Zeller C (2010). Untersuchung der Phylogenie von Tardigraden anhand der Genabschnitte 18S rDNA und Cytochrom c Oxidase Untereinheit 1 (COX I). MSc, Technische Hochschule Wildau, p. 105.